87. (Original) A compound according to Claim 11 wherein n is 2; R_6 is H; m is 1; r is 1; and R_4 and R_5 are each hydrogen.

88. (Original) A compound according to claim 64 or 87 wherein R_1 is 4-phenoxyphenyl.

REMARKS

Reconsideration of the Final Office Action mailed March 14, 2005, (hereinafter "instant Office Action",) entry of the foregoing amendment and withdrawal of the rejection of claims 1-35, 37-40, 44, 45, 47 and 51-88, are respectfully requested.

In the instant Office Action, claims 1-35, 37-40, 44, 45, 47 and 51-88 are listed as pending, and claims 1-35, 37-40, 44, 45, 47 and 51-88 are listed as rejected.

On page 25 claim 16 has been amended to correct a typographical error.

The Examiner has not repeated the rejection of claims 1-32 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention, with respect to allegedly not being enabling for the scope of the compounds claimed because only one compound has been made. Therefore, Applicants presume that the arguments and amendments submitted in the Reply filed December 23, 2004 was persuasive and the rejections have been withdrawn. Applicants respectfully request that the Examiner advise Applicants if this is not correct.

The Examiner has maintained the rejection of claims 1-35, 37-40, 44, 45, 47 and 51-88 under 35 U.S.C.§112, second paragraph, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicants respectfully traverse this rejection and maintain the arguments presented in the Replies filed December 23, 2004, May 17, 2004, March 15, 2004, July 28, 2003 and December 32, 2004. The Examiner acknowledges the definition pointed out by Applicants in the reply filed December 23, 2004 and states that "[a]pplicants should simply include this definition into the claims because C_1-C_6 is a well known prefix which appears before the terms alkyl, alkenyl, alkynyl which is here missing." As the Examiner has acknowledged, because C_1-C_6 is a well known prefix which appears before

the terms alkyl, alkenyl, alkynyl and thus is it not necessary for Applicants to incorporate the definition from the specification into the claims. As further proof that the term "C_1-C_6" is understood by one of ordinary skill in the art, Applicants attach herewith as Exhibit A a copy of U.S. Patent 6,660,744, issued December 9, 2003 in which claim 1, column 385, lines 23-24 read

"Z^{110} is a covalent bond, or an optionally substituted (C1-C6) which is optionally substituted...". Thus, the rejection is inconsistent with current USPTO practice and should be withdrawn.

Based upon the foregoing, the rejection of claims 1-35, 37-40, 44, 45, 47 and 55-88 under 35 U.S.C.§112, second paragraph, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention, is obviated and should be withdrawn.

The Examiner has maintained the rejection of claims 33-35, 37-40, 44, 47 and 51 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention. Applicants respectfully traverse this rejection and maintain the arguments presented in the Replies filed on December 23, 2004, May 17, 2004, March 15, 2004, July 28, 2004 and December 23, 2004.

On page 3 of the instant Office Action, the Examiner states:

> Applicants were unable to say who is in need of inhibiting any and all of the over 400 known kinases. The how to use portion of the statute has not been addressed. This means that Applicants must teach the skilled practitioner, in this case a physician, how to treat a given subject. The physician clearly must know what disease and what symptoms are to be treated.

As Applicants pointed out in the Reply filed December 23, 2004, on page 22, lines 7-20 of the instant specification Applicants state:

> In particular, compounds of this invention are useful as inhibitors of tyrosine kinases that are important in hyperproliferative diseases, especially in cancer and in the process of angiogenesis. For example, certain of these compounds are inhibitors of such receptor kinases as KDR, Flt-1, FGFR, PDGFR, c-Met, TIE-2 or IGF-1-R. Since certain of these compounds are anti-angiogenic, they are important substances

> for inhibiting the progression of disease states where angiogenesis is an important component. Certain compounds of the invention are effective as inhibitors of such serine/threonine kinases as PKCs, erk, MAP kinases, cdks, Plk-1 or Raf-1. These compounds are useful in the treatment of cancer, and hyperproliferative disorders. In addition, certain compounds are effective inhibitors of non-receptor kinases such as those of the Src (for example, Lck, blk and lyn), Tec, Csk, Jak, Map, Nik and Syk families. These compounds are useful in the treatment of cancer, hyperproliferative disorders and immunologic diseases.

Further, as the Examiner acknowledges on page 3 of the instant office action, Applicants list among the conditions to be treated leukemia, HIV and sarcoma. Thus, a patient presenting with any of these diseases or disorders would be in need of inhibition of tyrosine kinases. With respect to the Examiner's statement that Applicants "...have given no specific dose, given no specific dosing regiment, given no specific route of administration" Applicants respectfully direct the Examiner's attention to M.P.E.P. §2163.07(b) which states:

> ...to comply with 35 U.S.C. 112, first paragraph, it is not necessary to 'enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect.

Applicants respectfully point out that claims 33-35, 37-40, 44, 47 and 51 contain no such limitation and thus Applicants should not be required to provide a specific dose or dosing regiment or a specific route of administration.

Nonetheless, Applicants respectfully direct the Examiner's attention to the Pharmaceutical Formulation section on pages 66-70 of the instant specification wherein Applicants describe routes of administration. Applicants particularly direct the Examiner's attention to page 72, lines 21-23 of the instant application wherein it states "The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician." Only the physician directing the care of a patient can determine the best course of treatment (route of administration, dosage) for a patient depending upon factors specific to each individual patient (weight, severity of disease). The specification clearly conveys to one skilled in

the art what is being treated by claim 33, who the subject is, how one can identify said subject, and how dosage, dosing regimen and route of administration can be determined.

Further, Applicants respectfully point out that with regard to enablement and utility, the Utility Guidelines state:

"An applicant need only make one credible assertion of specific utility for the claimed invention to satisfy §101 and §112; additional statements of utility, even if not "credible" do not render the claimed invention lacking in utility.

With respect to the Examiner's comment that Applicants have not responded regarding claims 35, 38-40 and 44 with regard to the list of conditions to be treated, Applicants respectfully point out that in the Reply mailed December 23, 2004, Applicants included a copy of the review article "Tyrosine kinases in disease: overview of kinase inhibitors as therapeutic agents and current drugs in clinical trials", Strawn et al., *Expert Opinion on Investigational Drugs*, (1998) 7(4):553-573, as Exhibit A, which addresses the Examiner's concern regarding the list of disorders enumerated in the claims. For the Examiner's convenience Applicants have attached herewith another copy of Strawn et al. as Exhibit B. In the introduction, on page 554, Strawn et al. state "[t]yrosine kinases are also involved in restenosis, psoriasis, angiogenic diseases, immunological disorders and many other disease states, although their role in causative function is not always understood." On page 565 Strawn et al. states "For example, it is clear that anti-angiogenic tyrosine kinase inhibitors will have broad applicability in diseases such as rheumatoid arthritis, ocular diseases of neovascularisation (macular degeneration, diabetic retinopathy), psoriasis and restinosis." As evidenced above, Strawn et al. implicates tyrosine kinases in numerous diseases.

The Examiner has cited *Brenner v. Mason* 148 USPQ 696 and quoted "a patent is not a hunting license. It is not a reward for the search, but compensation for its successful conclusion." Applicants respectfully point out that in the instant application Applicants have taught how to make and use the compounds of the invention as well as provided assays which can be used to test the effectiveness of each compound as a kinase inhibitor. Applicants have taught how to use the compounds of the instant invention in the Pharmaceutical Formulation section of the application on pages 66, line 10 through page 70, line 30. Applicants have taught that there are diseases which can be affected by

inhibition of certain kinases at page 55, line 25 to page 56, line 24 and written claims drawn to using the instant compounds to treat said diseases. Applicants have provided a detailed explanation of how, using the compounds of the instant invention, the inhibition of protein kinase activity can be used in a therapeutic context.

The Examiner also cites *In re Diedrich* 138 USPQ at 130, quoting:

> We do not believe that it was the intention of the statutes to require the Patent Office, the courts, or the public to play the sort of guessing game that might be involved if an applicant could satisfy the requirements of the statutes by indicating the usefulness of a claimed compound in terms of possible use so general as to be meaningless and then, after his research or that of his competitors has definitely ascertained an actual use for the compound, adducing evidence intended to show that a particular specific use would have been obvious to men skilled in the particular art to which this use relates.

The Examiner has confused the requirement for utility versus the requirement for enabling how to make and how to use. The Examiner's rejection is not consistent with the Utility Guidelines issued by the U.S.P.T.O., which state "[When] applicant discloses a specific biological activity and reasonably correlates that activity to a disease condition...[it] would be sufficient to identify a specific utility for the compound". The Examiner's rejection seeks proof that a compound of formula I is effective in inhibiting one or more protein kinase activities. However, such evidence is not required for patentability. Applicants respectfully direct the Examiner's attention to M.P.E.P. 2107.03 V, which states:

> Thus, while an applicant may on occasion need to provide evidence to show that an invention will work as claimed, it is improper for Office personnel to request evidence of safety in the treatment of humans, or regarding the degree of effectiveness. See *In re Sichert*, 566 F.2d 1154, 196 USPQ 209 (CCPA 1977); *In re Hartop*, 311 F.2d 249, 135 USPQ 419 (CCPA 1962); *In re Anthony*, 414 F.2d 1383, 162 USPQ 594 (CCPA 1969); *In re Watson*, 517 F.2d 465, 186 USPQ 11 (CCPA 1975); *In re Krimmel*, 292 F.2d 948, 130 USPQ 215 (CCPA 1961); *Ex parte Jovanovics*, 211 USPQ 907 (Bd. Pat. App. & Inter. 1981).

35 U.S.C. §101 is concerned with whether an invention is useful. 35 U.S.C. §112, first paragraph, on the other hand, is concerned with how to make and use the invention. The Examiner is also focusing on "the compound", not "the invention". Applicants have

taught how to make and use the instant invention. Nevertheless, Applicants respectfully point out that the Utility Guidelines state the following:

> Courts have repeatedly found that the mere identification of a pharmacological activity of a compound that is relevant to an asserted pharmacological use provides an "immediate benefit to the public" and thus satisfied the utility requirement. As the CCPA held in Nelson v. Bowler:
>
> > Knowledge of the pharmacological activity of any compound is obviously beneficial to the public. It is inherently faster and easier to combat illnesses and alleviate symptoms when the medical profession is armed with an arsenal of chemicals having known pharmacological activities. Since it is crucial to provide researchers with an incentive to disclose pharmacological activities in as many compounds as possible, we conclude that adequate proof of any such activity constitutes a showing of practical utility.
>
> Similarly, courts have found utility for therapeutic inventions despite the fact that an applicant is at a very early stage in the development of a pharmaceutical product or therapeutic regimen based on a claimed pharmacological or bioactive compound or composition.
>
> Where an applicant has established utility for a species that falls within a[n] identified genus of compounds and presents a generic claim covering the genus, as a general matter, that claim should be treated as being sufficient under §101.

Applicants have provided assays to be used to measure inhibition activity of the instantly claimed compounds. Applicants have disclosed various utilities for the instantly claimed compounds. Therefore, Applicants have met the burden of enabling the instant invention.

Based upon the foregoing, the rejection of claims 33-35, 37-40, 44, 47 and 51 under 35 U.S.C. §112, first paragraph, is obviated and should be withdrawn.

The Examiner has maintained the rejection of claims 1-40 and 44-88 under 35 U.S.C. §103(a) as allegedly being unpatentable over Altmann et al. (WO 97/49706). Applicants respectfully traverse this rejection. Applicants maintain the arguments

presented in the Replies filed August 26, 2002, November 26, 2002, July 26, 2003, March 15, 2004, May 17, 2004 and December 23, 2004.

The Examiner alleges that "the reference teaches a generic group of substituted 7-amino-pyrrolo[3,2-d]pyrimidine derivatives which embraces applicants' claimed compounds". The Examiner points to Example 72 on page 35 as the closest prior art because it contains a 4-Ph-OH substituent over the 4-Ph-OPh group of the instant compound at R_1.

The Examiner alleges that the reference teaches 4-amino-7H-pyrrolo[2,3-d]pyrimidin-5-yl compounds having an optionally substituted phenyl as the 5-substituent which is further attached to $-A-R_5$ wherein A is $-NH-SO_2-$ and R_5 is optionally substituted phenyl, etc. The Examiner further states that the instant claim differs by reciting specific species that fall within the reference genus. It is well established that a prior art genus does not make a basis for a *prima facie* case of obviousness because the species falls into it. In re Baird, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994) ("The fact that a claimed compound may be encompassed by a disclosed generic formula does not by itself render that compound obvious"); In re Jones, 958 F2d 347, 350, 21 USPQ2d 1941, 1943 (Fed. Cir. 1992).

The Examiner has not established by clear and convincing evidence that one of ordinary skill in the art would have been motivated to select example 72 out of the 107 compounds disclosed in Altmann et al. as a lead compound. *Yamanouchi*, 231 F.3d at 1344; see also *Dillon*, 919 F.2d at 602 (finding that *prima facie* obviousness is established "where the prior art gives reason or motivation to make the claimed compositions"). In In re Baird, supra, the court found:

> ...the generic diphenol formula disclosed in Knapp contains a large number of variables, and we estimate that it encompasses more than 100 million different diphenols, only one of which is bisphenol A. While the Knapp formula unquestionably encompasses bisphenol A when specific variables are chosen, there is nothing in the disclosure of Knapp suggesting that one should select such variables.

In much the same way, the genus of Altmann encompasses thousand of compounds and like In re Baird, when specific variables are chosen the genus of Altmann overlaps with Applicants' genus. However, one or ordinary skill in the art could select any number of

variables and arrive at a different genus which would also overlap with Altmann. The Examiner is using hindsight instead of pointing to the motivation that one would select precisely the right variables to arrive exactly at Applicants' genus based upon Altmann's disclosure. Merely because the genuses overlap does not make Applicants' genus obvious over Altmann. The Examiner has not pointed to any motivation or suggestion in Altmann to arrive at exactly Applicants' genus.

In re Baird further found:

> The diphenols that Knapp specifically discloses to be 'typical, ' 'preferred,' and 'optimum' are different from and more complex than bisphenol A, we conclude that Knapp does not teach or fairly suggest the selection of bisphenol A. See *In re Bell*, 991 F.2d 781, 26 U.S.P.Q.2d (BNA) 1529 (Fed. Cir. 1993).

Like Knapp, Altmann discloses preferred examples and on page 8 states "[t]he invention relates especially to the specific compounds described in the Examples and to salts hereof." The majority of the examples in Altmann contain a phenyl substituted with a simple moiety such as methoxy in the 5-position. Thus, in the instant case Applicants' have disclosed a genus which is different from and more complex than that of Altmann.

Similarly to In re Baird, the reference discloses a genus with a number of variables which encompass a large number of possible compounds. Again, like In re Baird, the reference does not provide any suggestion or motivation to arrive at exactly Applicants' genus. Merely because the genuses overlap does not make Applicants' genus obvious over Altmann.

Based upon the foregoing, the rejection of claims 1-40 and 44-88 under 35 U.S.C. §103(a) over Altmann et al. (WO 97/49706) is obviated and should be withdrawn.

Based upon the foregoing, Applicants believe that claims 1-35, 37-40, 44-45, 47 and 51-88 are in condition for allowance. Prompt and favorable action is earnestly solicited.

If the Examiner believes that a telephone conference would advance the condition of the instant application for allowance, Applicants invite the Examiner to call Applicants' agent at the number noted below.

Respectfully submitted,

Date: August 11, 2005

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Exhibit B

Expert Opinion on Investigational Drugs

http://www.ashley-pub.com

Review

Tyrosine kinases in disease: overview of kinase inhibitors as therapeutic agents and current drugs in clinical trials

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Tyrosine kinases, first described as oncogenes, have been shown to play a role in normal cellular processes. Aberrations in tyrosine kinase activity lead to disease states. For fifteen years it has been postulated that the inhibition of tyrosine kinases may have therapeutic utility and the design and testing of inhibitors have been major focuses of research and development in both academic institutions and pharmaceutical companies. While early research focused on developing chemical entities that mimic phosphotyrosine, later research has focused on developing competitive adenosine triphosphate (ATP) inhibitors with various levels of selectivity on kinase targets. This review focuses on a discussion of tyrosine kinases thought to be important in disease, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial cell growth factor (VEGF), epidermal growth factor (EGF) receptors, HER-2 and Src. In addition, the classes of inhibitors designed to affect these targets and that have overcome research and development challenges and entered clinical trials are discussed. These include isoxazole, quinazoline, substituted pyrimidines and indolinone compounds, all of which are in clinical trials or near clinical development by SUGEN, Zeneca, Novartis, Pfizer and Parke-Davis. A summary of the chemistry and activity of these agents is provided.

Keywords: *angiogenesis, cancer, fibrosis, growth factors, indolinones, isoxazoles, psoriasis, pyrimidines, quinazolines, restenosis, tyrosine kinases*

Exp. Opin. Invest. Drugs (1998) 7(4):553-573

1. Introduction

Tyrosine kinases are enzymes that regulate signal transduction in cells, leading to mitogenesis, differentiation, migration, apoptosis and many other cellular functions. They exist as two major structural types, transmembrane receptors and cytoplasmic proteins. The receptor tyrosine kinases typically have an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase domain. Upon ligand binding, they dimerise and undergo a conformational change that activates the kinase, leading to transphosphorylation. Other substrates, adapter proteins and even cytosolic tyrosine kinases bind to the phosphorylated tyrosines of the receptor leading to a cascade of events that results in the final cellular function. Cytosolic tyrosine kinases may be activated by phosphorylation, as in the case of AKT, or by dephosphorylation, as occurs with Src family members.

As tyrosine kinases are involved in so many cellular functions, aberrant activity can lead to disease states. Such aberrant activity may be the result of

overactivation, as well as underutilisation of the signalling pathways. Many oncogenes found in cancers are derived from tyrosine kinase genes that have been deregulated, leading to constitutive activity [1,2]. Overexpression or activation of wild-type tyrosine kinases can also lead to cancer. Abnormal activation may result from an autocrine loop in which a growth factor and its receptor are expressed in the same cell, leading to continuous signalling [3]. Tyrosine kinases are also involved in restenosis, psoriasis, angiogenic diseases, immunological disorders and many other disease states, although their role in causative function is not always understood.

Type II diabetes is an example where too little signalling through a tyrosine kinase-mediated pathway leads to disease. In this case, lack of insulin receptor signalling results in a deficiency of cellular glucose uptake, causing hyperglycaemia. Other examples where a deficiency in signalling leads to pathological conditions include neuropathies, anaemias and immune suppression. This review will focus only on the overactivation of tyrosine kinases in disease and the development of tyrosine kinase inhibitors as therapeutic agents, which has led to a fast growing and competitive area of research (reviewed in [4]). Many tyrosine kinase inhibitors with different structural types have been introduced and these have recently been reviewed [5-9]. Here we discuss the major tyrosine kinases that have been validated as playing a role in disease and the advances that have been made in developing agents that inhibit them, with emphasis on the kinase inhibitors that have entered clinical trials.

2. Tyrosine kinases in disease states

2.1 Platelet-derived growth factor receptor

Platelet-derived growth factor (PDGF) is a major mitogen and chemoattractant for fibroblasts, smooth muscle cells and glial cells (reviewed in [10]). It exists as disulfide-linked homo- and heterodimers of A-chain and B-chain, resulting in three isoforms. These isoforms have differential binding affinities for the receptors, which are made up of α- and β-subunits. This family of receptors is characterised by Ig-like loops in the extracellular domain and a split kinase domain (reviewed in [11]). The receptors exist as homo- and heterodimers depending on which PDGF isoform is present. Signalling through both receptors causes phosphatidylinositol turnover, calcium flux and membrane ruffling, leading to cellular migration and proliferation.

The specific role of each receptor type is not fully understood, but recent studies of targeted mutagenesis in mice prove that, at least in development, there are distinct functions for the two receptor types. For example, mice embryos deficient in the α-receptor or the β-receptor do not survive until birth and embryos of both show evidence of bleeding, but each also causes specific defects [12,13]. Deficiency of the α-receptor causes severe bone abnormalities [13], whereas deficiency of the β-receptor results in kidney defects in embryos [12]. Furthermore, the β-receptor appears to play a role in angiogenesis by regulating the pericytes that surround vessels [14]. PDGF-AA and the α-receptor are required for gliogenesis during development [15]. PDGF is secreted from type-1 astrocytes [15] and neurones [16] and induces proliferation and differentiation of O2-A progenitors. In adults, PDGF plays a role in wound healing. Its chemotactic activity attracts fibroblasts, smooth muscle cells, monocytes and neutrophils to the site of injury (reviewed in [17]).

PDGF and its receptors have been implicated in a number of disease states including cancer. The PDGF B-chain gene was the first proto-oncogene to be identified when it was found to be the cellular homologue of v-*sis*, the transforming gene of simian sarcoma virus [18,19]. Subsequently, PDGF has been detected in many tumour cell lines, including breast [20] and colon cancers [21], and melanoma [22]. PDGF and its receptor are co-expressed in numerous tumour cell lines, such as sarcomas [23] and gliomas [22,24,25]. More importantly, the presence of PDGF and its receptors in tumour biopsies has been shown. In ovarian tumours, PDGF and the α-receptor were detected in malignant tumours by immunohistochemical staining, but they were not present in benign tumours or normal tissue [26]. Biopsies from human gliomas have been analysed by *in situ* hybridisation [27-29]. In high grade glioma, PDGF A-chain and PDGF α-receptors were co-expressed in tumour cells; they possibly function through an autocrine loop in such tumours. The β-receptor was expressed on endothelial cells of the tumours and B-chain was detected in both tumour and endothelial cells; paracrine and autocrine stimulation of receptors may occur in this situation.

The inhibition of PDGF signalling in tumour models has also been used to study the role of PDGF receptors in cancer. In a rat glioma cell line, introduction of a truncated PDGF β-receptor inhibited both *in vitro* growth and sc. tumour growth in athymic mice through a dominant-negative mechanism [30]. Similar results were found with mutant forms of PDGF A-chain [31,32]. These findings, along with the expression patterns, support the theory that PDGF and its receptors play a role in many types of human cancers.

PDGF has also been implicated in atherosclerosis [33] and restenosis [34]. *In vitro*, it is a chemoattractant and mitogen for smooth muscle cells, which are deposited in vessels in both conditions. In 30 - 40% of patients undergoing angioplasty to remove atherosclerotic plaques, restenosis occurs within six months. Balloon injury to the carotid artery of rats is utilised to mimic the process of restenosis, although this model has shortcomings. PDGF A-chain was quickly up-regulated in smooth muscle cells following injury and PDGF β-receptor mRNA increased gradually during the chronic phase of neointimal formation [35]. Furthermore, accumulation of smooth muscle cells in this model can be blocked by an antibody against PDGF [36]. It appears that PDGF stimulates the migration of smooth muscle cells, but not their proliferation. Restenosis is a complex process involving many factors, but PDGF plays a role in at least one aspect of it.

2.2 Fibroblast growth factor receptor

Fibroblast growth factor (FGF) receptors are related to PDGF receptors in that they have extracellular Ig-domains and a split kinase domain (reviewed in [37]). The receptors are coded for by four different genes and types 1 and 2 also have multiple isoforms due to alternative splicing. At least ten different forms of FGF have been identified [38-40]. All forms bind to heparin and heparan sulfate on cell surfaces. This association increases the affinity of FGF for its receptors. FGFs also bind to the extracellular matrix, which may serve as a storage site. FGF-1 and -2, also known as acidic and basic FGF, are the best characterised. Neither contains a signal sequence and the mechanism of secretion is not clear. Both bind to all four receptor types and stimulate the growth and migration of a number of cell types, including fibroblasts and endothelial cells.

As with PDGF, FGF plays a role in embryonic development. FGF-3 (INT-2) and FGF-4 (K-FGF/HST-1) are only expressed in embryos at specific time points [38]. FGF-6, which only binds to the type-4 receptor and FGF-2, appears to be involved in muscle development [38,41]. The expression pattern of FGF receptor-1 in mouse embryos is consistent with involvement in mesodermal patterning. Targeted mutagenesis of its gene caused a lethal phenotype in homozygotes with the embryos showing aberrant mesodermal patterning [42]. To investigate the role in skin development of the FGF receptor-2, which is the receptor for FGF-7 (keratinocyte growth factor), transgenic mice were developed that express a truncated form of the receptor in their epidermis [43]. The mutant receptor inhibited the wild-type receptor and caused abnormalities that suggest this receptor is required for normal keratinocyte differentiation. FGF receptor-3 is apparently involved in skeletal growth. Mutations in its gene have been identified in two types of dwarfism, achondroplasia [44,45] and thanatophoric dysplasia [46].

In the adult, the FGF receptor is involved in wound healing. FGF-2 increases the formation of granulation tissue and induces migration of fibroblasts and endothelial cells to the wound site [47]. FGF-7 is up-regulated by over 100-fold within 24 h of wounding and stimulates growth of keratinocytes [48]. Transgenic mice expressing an inhibitory form of FGF receptor-2 (the receptor for FGF-7) in their epidermis had delays in wound healing [49]. FGF also participates in wound healing by stimulating angiogenesis. FGF-1 and FGF-2 induce the release of proteases, and migration and proliferation of endothelial cells, all of which are required for angiogenesis (reviewed in [50,51]). FGFs have been shown to induce angiogenesis in a number of *in vivo* systems, including the chorioallantoic membrane of chicken embryos, corneas of mice and implants in rodents.

Just as FGF has similar biological functions to PDGF, it is also involved in the same diseases. FGF-1 and -2 and their receptors have been identified in a variety of tumour types. A human renal cell carcinoma cell line [52] and two human prostate tumour cell lines [53] produce FGF-2. In a panel of human oesophageal cancer cell lines, FGF-2 and FGF receptor-1 mRNAs were co-expressed; possibly an autocrine loop drives their growth [54]. In the skin of melanoma patients, high FGFR-1 expression was seen in invading melanoma cells and stroma, but not in the endothelial cells [55]. Analysis of astrocytomas showed that mRNA expression for type 1 and type 2 FGF receptors changes as tumours progress to higher grades [56].

FGFs and their receptors are expressed in tumour endothelium, as well as tumour cells. They are likely to play a role in both the proliferation of tumour cells and tumour angiogenesis.

Neutralising antibodies against FGF-2 have been used to confirm that it has a functional role in cancer. An antiFGF-2 antibody blocked mitogenesis of SC115 mouse mammary carcinoma cells in response to FGF-1 [57]. Similarly, a monoclonal FGF-2 antibody inhibited the growth of U-87MG and T98G human glioblastoma cells in culture and in nude mice [58].

FGF also stimulates vascular smooth muscle cells to migrate and proliferate in restenosis [34]. FGF-1 and FGF-2 are released from dying smooth muscle cells during angioplasty. Furthermore, mRNA for FGF-2 and FGF receptor-1 are up-regulated in smooth muscle cells after injury and may act by an autocrine mechanism [59]. A neutralising antibody against FGF-2 inhibited early smooth muscle cell proliferation in a balloon injury model, although it did not reduce the size of the intimal lesion [60]. As with PDGF, FGF is important for restenosis, but it does not act alone.

2.3 Flk-1/KDR

Flk-1 is structurally related to PDGF and FGF receptors with seven immunoglobulin-like sequences in the extracellular domain and a split tyrosine kinase domain. It is a receptor for vascular endothelial cell growth factor (VEGF) [61,62]; KDR is its human homologue [63]. Other members of the family are Flt-1 [64,65], which also binds VEGF, and Flt-4 [66]. Flk-1/KDR and Flt-1 are expressed primarily on vascular endothelium and Flt-4 is expressed on lymphatic endothelium. VEGF stimulates growth of endothelial cells during the process of angiogenesis, the sprouting of new blood vessels from pre-existing vessels (reviewed in [67]). It was also independently isolated as vascular permeability factor (VPF) (reviewed in [68]). VEGF has four different splice variants and exists as a disulfide-linked homodimer with structural similarities to the PDGFs. Recently, two new members of the VEGF family have been identified, VEGF-B and VEGF-C. VEGF-B stimulates the growth of endothelial cells, but its receptor, or receptors, is not yet known [69]. VEGF-C, also known as vascular endothelial growth factor-related protein, was identified as a ligand for Flt-4 [70,71], but it also binds to Flk-1/KDR. There is some evidence that co-expression of two VEGF family members in the same cell type leads to the formation of heterodimers. The functions of such proteins remain to be elucidated.

VEGF and its receptors are clearly involved in angiogenesis during development. Binding of [^{125}I]-labelled VEGF [72] and *in situ* hybridisation have been used to show that VEGF [73] and its receptors [62] are expressed in vascular endothelium in mouse embryos. Furthermore, targeted deletion of the Flk-1 gene resulted in embryos with a defect in haematopoietic and endothelial cell development that led to a lack of organised blood vessels [74]. In normal adults, angiogenesis only occurs in wound healing, corpus luteum formation and pregnancy. Correspondingly, Flk-1 was not found to be expressed in vessels of normal adult mice [62].

Angiogenesis is required for tumours to grow beyond a minimum volume and to switch to a neoplastic phenotype [75]. The roles of VEGF and Flk-1/KDR are well defined in this process. VEGF is secreted by a number of human tumour cell lines in culture, such as glioma [76], melanoma [77] and epidermoid carcinoma cells [78]. More importantly, VEGF transcripts or protein have been identified by *in situ* hybridisation or immunohistochemistry in primary gliomas [79,80], haemangioblastomas [81], and breast [82-84], colon [85,86] and renal cell tumours [87]. Like VEGF, mRNA for Flk-1/KDR has been detected in tumours, such as gliomas [79,80], haemangioblastomas [81], colon cancer [86] and adenocarcinomas [85]. In all cases, the receptors were detected on the endothelial cells of the vessels and not the tumour cells. This supports a paracrine mechanism in which VEGF secreted from tumour cells stimulates proliferation of endothelial cells.

A number of animal models have been developed to investigate the function of VEGF and Flk-1/KDR in tumour angiogenesis. The introduction of antisense constructs against VEGF into rat C6 glioma [88] and human U87MG glioblastoma cells lines [89] reduced their sc. growth in athymic mice, as well as the degree of neovascularisation. Monoclonal antibodies against VEGF have also been utilised to inhibit the sc. growth of several human tumour cell lines in athymic mice [90,91]. Truncated Flk-1 was used to study the capacity of Flk-1 to act as a modulator of tumour growth in animal models. Athymic mice were co-implanted with tumour cells and virus-producing cells that carry the mutant *flk-1* gene [92,93]. This allowed the introduction of mutant receptor into endothelial cells, where it acted by a dominant-negative mechanism to block

activation of Flk-1. By this method, the sc. growth of a variety of human, rat and mouse tumour cells was inhibited. The vessel density was also reduced in the small tumours that did form, which confirmed the connection between Flk-1, angiogenesis and tumour growth.

Angiogenesis occurs in other disease states, such as haemangioblastoma formation, psoriasis and diabetic retinopathy. VEGF and Flk-1 have been shown to play a role in these situations, as well as in cancer [67]. In two studies, haemangioblastomas, which are highly vascularised tumours, were shown to express VEGF, Flk-1/KDR and Flt-1 [81,94]. All three proteins were also found to be highly expressed in psoriatic skin, but not in normal skin [95]. Psoriatic lesions are hyperpermeable as well as highly vascularised. VEGF is up-regulated in the ocular fluid of patients with diabetic retinopathy [96]. It is apparently induced by hypoxia, which occurs as a result of vessel damage in the retina. VEGF signalling through Flk-1/KDR is involved in a number of angiogenic diseases, but occurs only rarely in normal adults. This makes Flk-1/KDR an ideal target for therapeutic intervention.

2.4 Epidermal growth factor receptor

The epidermal growth factor (EGF) receptor was cloned in 1984 by Ullrich *et al.* [97]. It has two cysteine-rich regions in the extracellular domain and a single kinase domain. There are three other known members of the EGF receptor family, HER-2, HER-3 and HER-4 (also known as erbB2, erbB3 and erbB4). Besides EGF, several other ligands bind to the EGF receptor: transforming growth factor-α (TGF-α), betacellulin, amphiregulin, heparin-binding EGF (HB-EGF) and neuregulin (also known as heregulin). EGF and betacellulin induce formation of EGF receptor heterodimers with the other family members, as well as EGF receptor homodimers. Likewise, betacellulin is a ligand for HER-4 as well as the EGF receptor and binds to heterodimers containing either receptor.

Unlike PDGF and FGF receptors, the EGF receptor is not absolutely required for development. Although deletion of the EGF receptor gene can be lethal in some genetic backgrounds, some strains of mice have survived up to three weeks after birth despite a deficiency in the EGF receptor [98,99]. The mice were born with eyes opened, rudimentary whiskers and epidermal defects. They also had abnormalities in kidney, brain, liver and the gastrointestinal tract; thus the EGF receptor is involved in the development of several organs.

Members of the EGF receptor family and its ligands are overexpressed, or expressed as an autocrine loop in many tumour types. Amphiregulin was found to be co-expressed with the EGF receptor in pancreatic cancer cells [100] and ovarian carcinoma specimens [101]. Ten out of 13 renal cell lines studied express TGF-α along with the EGF receptor [102]. HB-EGF was expressed in an autocrine loop in gastric cancers and cell lines [103] and hepatocellular carcinomas [104]. In a study of primary breast tumours, 59% of the samples expressed the EGF receptor and its expression correlated with nonresponsiveness to hormone therapy [105]. In most cases, overexpression of the EGF receptor does not result from gene amplification, but the gene was amplified in some glioblastomas [106].

Not only is the EGF receptor present in many tumours, it may be required for tumour cell growth. This was shown with an inhibitory antibody against the receptor, which blocked the growth of tumour cell lines, both *in vitro* and as xenografts in athymic mice [107,108]. Furthermore, an antisense oligonucleotide for amphiregulin inhibited the growth of a pancreatic cancer cell line [100]. The frequent occurrence of the EGF receptor in an autocrine loop in cancer makes it a good target for chemotherapy.

Several lines of evidence suggest a role for the EGF receptor system in psoriasis, a disease characterised by disregulation of keratinocyte cell growth with epidermal hyperplasia. The TGF-α/EGF receptor system is an excellent example of autocrine activation leading to epidermal keratinocyte proliferation. TGF-α is produced by keratinocytes in normal human epidermis and cultured keratinocytes [109,110]. However, it is overexpressed in psoriatic epidermis as determined by immunohistochemistry and mRNA assays [109-115]. Studies using [^{125}I]-EGF have shown that the normal basilar distribution of EGF receptors in epidermal keratinocytes was markedly altered in *psoriasis vulgaris* where they were also observed in the upper keratinocyte compartment [115,116]. In a study on benign epidermal dermatoses, EGF receptor expression throughout the epidermis returned to a basal layer distribution when the lesion resolved [117]. Finally, it has been shown that other cytokines and growth factors, such as IFN-γ, TNF-α, IL-8 and IGF-1, induced expression of TGF-α, EGF receptor or both [118,119]. These data suggest that inhibition of EGF

receptor signalling may provide therapeutic benefit in psoriasis by controlling the hyperproliferation of psoriatic keratinocytes.

2.5 HER-2

The rat homologue of HER-2, Neu, was originally identified as a transforming protein in ethylnitrosourea-treated rats [120]. The oncogene has a point mutation in the transmembrane domain that codes for a mutant receptor-like protein that dimerises constitutively [121], resulting in activation of the tyrosine kinase. The human homologue, HER-2, was found in an effort to clone EGF receptor-related genes [122,123]. It remains an orphan receptor despite years of effort to identify a ligand. It is constitutively activated when overexpressed even without the activating mutation found in Neu [124,125]. It forms heterodimers with the EGF receptor in the presence of EGF [126] and with HER-3 and HER-4 in the presence of neuregulins [127,128]. HER-2 is activated when heterodimerised with other family members and is the preferred partner for the other receptors [129].

The normal role of HER-2 is not clear. It may regulate the activities of EGF and the neuregulins by interacting with their receptors. Potentially, it is involved in differentiation and mitogenesis of many cell types. Its role in cancer is much better understood. It has been found to be overexpressed in about 30% of breast and ovarian cancers and expression has been correlated with poor prognosis (reviewed in [130]). In some cases, overexpression is a result of gene amplification. Recently, the HER-2 gene was also found to be amplified in many prostate carcinomas and this correlated with tumour grade and disease recurrence [131]. Higher expression was also seen in colorectal adenocarcinoma than in benign lesions [132]. As well as contributing to proliferation of tumour cells, HER-2 may protect them from the immune system by inducing resistance to TNF-α [133] and lymphokine-activated killer cells [134].

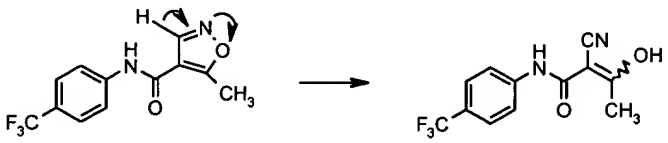
Extensive research has been done with inhibitory antibodies against HER-2. In NIH 3T3 cells transformed by Neu, a monoclonal antibody down-regulated Neu and reverted the transformed phenotype [135]. In AU-565 human breast cancer cells, an antiHER-2 antibody induced differentiation [136]. Antibodies were also effective at inhibiting the growth of sc. tumours resulting from implantation of HER-2-expressing NIH 3T3 cells in athymic mice [136,137]. These results confirm that HER-2 plays a role in cancer and that it is an excellent target for intervention. Indeed, a humanised monoclonal antibody against HER-2 is currently in clinical trials by Genentech. In a small Phase II trial, 11.6% of patients responded and 37.2% had stable disease after ten doses [138]. Phase III studies are expected to be completed this year.

2.6 Src Family

The first oncogene to be discovered was v-*src*, the transforming gene of the Rous sarcoma retrovirus. Its cellular homologue, c-*src*, was later found and both were determined to code for tyrosine kinases. There are now nine known members of the Src family: Src, Fyn, Yes, Lck, Lyn, Fgr, Hck, Blk and Yrk. Unlike the receptor tyrosine kinases, the Src family of tyrosine kinases lack ligand binding and transmembrane domains. They contain Src homology 2 and 3 domains, known as SH2 and SH3 domains, which mediate interactions within the protein and with other proteins. These domains are also found in many other proteins and their specific binding sequences are well known [139]. SH2 domains bind to phosphotyrosine residues with flanking amino acids that are specific for the particular SH2 sequence. SH3 domains bind to proline-rich regions. Each Src family member also has a unique domain near the amino terminus and is associated with the membrane by myristilation.

Src family members are regulated by phosphorylation of a tyrosine in the carboxy-terminal tail. Dephosphorylation of this tyrosine allows a conformational change that leads to kinase activation. Recently, the crystal structure of inactive Src was analysed to reveal in more detail how Src is regulated [140]. Not only does the SH2 domain associate with the phosphotyrosine at 527, but the SH3 domain locks the protein in the inactive state by associating with the linker between the SH2 and kinase domains. Although Src family members are not activated directly by ligands, they are activated in response to PDGF, EGF and other growth factors. In the case of the PDGF receptor, Src family members bind through their SH2 domains to two tyrosine residues in the juxtamembrane domain of the PDGF receptor [141-143]. Src is activated and, in turn, phosphorylates tyrosine 934 of the PDGF β-receptor [144]. PDGF receptors mutated at this site have decreased mitogenic responses to PDGF-BB. Furthermore, introduction of a kinase inactive Fyn construct into NIH 3T3 cells inhibits PDGF-induced mitogenesis by blocking the association of active Src family members to the PDGF receptor [145].

Figure 1: Structure of SU101 and its major metabolite SU0020.



SU101
Leflunomide
HWA 486

SU0020
A771726
RS-61980-00

Src is also involved in signalling by EGF receptor family members. Co-expression of the EGF receptor and Src in murine fibroblasts was used to show that Src associates with the EGF receptor and that EGF-induced mitogenesis was increased in the presence of Src [146]. Furthermore, the co-expressing cells were able to grow in soft agar and athymic mice. In another system with activated Neu expressed under an inducible promoter, it was shown that Src binds to phosphorylated Neu increasing its kinase activity [147]. Direct interaction between Src and the EGF receptor was not detected; they may associate through Neu.

Despite the fact that Src is involved in mitogenic signalling from growth factor receptors, deletion from mouse embryos is not lethal [148]. This may be because Fyn, Yes or other family members can substitute for Src. The mutant mice died within a few weeks of birth and were found to have impaired osteoclast function, indicating that Src may play a role in bone remodelling.

Src family members are also thought to be involved in cancer, particularly of the colon. In colon carcinoma cell lines and biopsy samples, Src was found to be highly activated compared to normal colonic mucosal cells and normal tissue [149]. Yes was also found to be elevated in another set of samples, while other family members, Lck, Fyn, Hck, Lyn and Fgr, were not [150]. It has also been shown that Yes kinase activity was higher in colonic adenomas that had signs indicating that they were at risk for malignancy [151]. In a recent study, antisense against Src was able to not only reduce the level of Src in the human colon tumour cell line HT 29, but also to reduce their proliferation rate *in vitro* and tumourigenesis in athymic mice [152]. Although both Src and Yes appear to play a role in colon cancer, only Yes was found to have elevated activity in a panel of melanoma cell lines compared to normal melanocytes [153]. Src family members may be able to substitute for each other in normal cells, but, in cancerous cells, only one or two members may contribute to the transformed phenotype.

3. Tyrosine kinase inhibitors

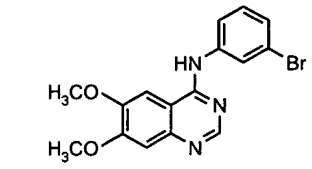
Several review articles have recently been published that describe the different classes of tyrosine kinase inhibitors [5-9]. In this section, the inhibitors that have overcome the preclinical challenges and entered clinical development will be discussed. Therefore, while kinase inhibitors, such as quinolines, quinoxalines, tyrphostins, biarylhydrazones, and natural products, such as erbstatin, genistein, herbimycin A and geldanamycin, have been important historically and served as the basis for much of the early development of kinase inhibitors, they will not be reviewed here.

3.1 SU101/leflunomide

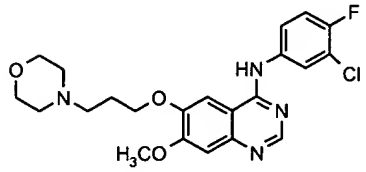
N-[(4-trifluoromethyl)-phenyl]-5-methylisoxazole4--carboxamide is in clinical trials by SUGEN (SU101) for cancer and Hoechst AG (Leflunomide) for rheumatoid arthritis. SUGEN has begun a Phase III trial in recurrent malignant glioma and Hoechst AG has completed or is near completion of its Phase III trial in rheumatoid arthritis. The compound has been studied by several laboratories, where activity has been ascribed to two areas, inhibition of tyrosine kinases and inhibition of pyrimidine biosynthesis. The isoxazole compound is rapidly converted *in vivo* to the metabolite, 2-cyano-3-hydroxy-N-(4-trifluoromethylphenyl)butenamide, which is known by several names as shown in **Figure 1**.

The literature describes activities associated with both the closed-ring parent compound and the open-ring metabolite, although most studies have focused on the metabolite. The open-ring structure has been reported to have effects on a number of tyrosine

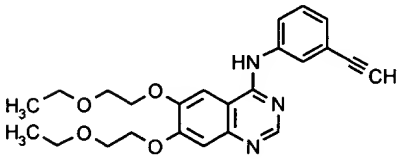
Figure 2: Quinazolines in Phase I clinical trials.



PD 153035/SU5271



ZD1839



CP 358 774

kinases, including Fyn and Lck, and tyrosine phosphorylation of the T-cell receptor ζ chain induced by antiCD3 monoclonal antibodies [154-156]. In addition, Mattar *et al.* found that phosphorylation of the EGF receptor was inhibited by the open-ring metabolite [157]. Elder *et al.* found inhibition of IL-2-induced tyrosine phosphorylation of JAK1 and JAK3, as well as tyrosine phosphorylation of the β-chain of the IL-2 receptor [158]. Shawver *et al.* showed that PDGF-mediated signalling was inhibited by SU101, including receptor phosphorylation, cell cycle, DNA synthesis, and tumour cell growth [159]. The latter studies focused on the parent, closed-ringed structure rather than the open-ring metabolite.

In addition to inhibiting tyrosine kinases, the open-ring metabolite has been shown to inhibit pyrimidine biosynthesis *via* inhibition of dihydroorotate dehydrogenase, the fourth enzyme of *de novo* pyrimidine biosynthesis [160-162]. The effects on pyrimidine biosynthesis can be overcome by the addition of exogenous uridine to cell cultures, to replenish the nucleotide pools [158,160,161]. However, the addition of uridine does not overcome the effects on proliferation due to antagonising tyrosine kinase activity [156,158,159]. Thus, the anti-inflammatory and anticancer properties of this compound are likely due to at least two separate and distinct activities. It has recently been shown [Lipsom *et al.*, manuscript in preparation] that the PDGF-mediated activity of SU101/leflunomide can be ascribed to the parent molecule, and the route of drug administration influences the ability to deliver its anticancer activity *in vivo*. Whereas broad activity was observed following parenteral administration, inhibition of PDGF-mediated tumour growth was not observed following oral administration, most likely due to conversion to the metabolite in the gastrointestinal tract. Thus, it appears that only parenteral administration of the compound retains its ability to inhibit PDGF-mediated cancers.

3.2 Quinazolines

Quinazolines have been studied extensively in several pharmaceutical companies and three are currently in clinical development (**Figure 2**). PD 153035 was published in 1994 [163], as a compound that inhibits the EGF receptor phosphorylation at picomolar concentrations and HER-2 at low micromolar concentrations, but only had effects on the PDGF and FGF receptors at 50 μM. Hence, this compound is a very selective tyrosine kinase inhibitor. This is in contrast to the structurally similar quinolines and quinoxalines, early tyrosine kinase inhibitors, which differ in the position and/or number of the nitrogens in the rings and show poor selectivity in comparison. Other structure-activity studies examined the distance between the amine and the phenyl group, and substituents on both the quinazoline and aniline moieties [164-166]. As with quinolines and quinoxalines, 6,7-dimethoxy-substituted quinazoline compounds were the most potent, although dihydroxy- and diamino-substituted quinazolines were also active [164,165]. Halogens in the meta position of the aniline group were the most effective at inhibiting the EGF receptor in kinase assays [165,166]. The combination of electron donating groups in the 6- and 7-positions and a halogenated aniline group has a 'supra-additive' effect, in which the potency was much greater than expected [165]. Growth of KB tumour cells was inhibited by quinazolines, and 4-(3-chloroanilino)quinazoline inhibited EGF-stimulated, but not PDGF- or IGF-stimulated, growth of other cell lines [166]. Not only is PD 153035 active in *in vitro* EGF receptor tyrosine kinase assays, but a single dose reduced the

phosphorylation of the EGF receptor in tumours derived from implantation of A431 cells in athymic mice [167]. Parke-Davis is not pursuing clinical trials of PD 153035 in cancer, but the compound is also known as SU5271 and is in Phase I clinical trials by SUGEN as a topical agent for the treatment of psoriasis. As discussed previously, the EGF receptor is thought to be a primary driver of keratinocyte proliferation through activation by either EGF or TGF-α. Since this compound is one of the most potent EGF receptor inhibitors known, it may provide proof-of-concept for the role of EGF in psoriasis.

ZD 1839 is under development by Zeneca for the treatment of cancer and is in Phase II studies. Like other quinazolines, it is a potent EGF receptor inhibitor, exhibiting an IC_{50} of 23 nM for tyrosine kinase activity and 80 nM for inhibition of KB cell proliferation [168]. It was shown to be a competitive ATP inhibitor with a K_i = 2.1 nM on purified EGF receptors. ZD 1839 showed excellent activity against xenografts in athymic mice. When administered orally at 10 mg/kg/day, a 50% reduction in the growth of A431 cells was observed. Activity was also shown against A549 (lung), Lovo (colon) and DU145 (prostate) tumours. Perhaps surprising, however, was the observation that 200 mg/kg/day caused regression of 1.5 g A431 tumours to undetectable sizes within two weeks, and tumour growth was suppressed for as long as 4 months. Tumour regression would not be expected for a cytostatic growth factor receptor inhibitor. However, regrowth occurred when treatment was suspended.

CP-358,774 is a quinazoline under clinical development by Pfizer [169,170]. It is also a potent EGF receptor tyrosine kinase inhibitor (IC_{50} = 2 nM) and shows greater than 1000-fold selectivity against other tyrosine kinases such as $pp60^{v\text{-}src}$, $pp145^{c\text{-}abl}$, the insulin receptor and the IGF-1 receptor. It has been shown to be a reversible ATP competitive inhibitor. Daily oral administration at 10 mg/kg to athymic mice bearing HN5 (head and neck tumour cells) xenografts resulted in 50% inhibition. For inhibition of growth of A431 derived tumours, a higher dose of CP-358,774 was needed (200 mg/kg/day). As well as inhibiting tumour cell growth, CP-358,774 induced apoptosis in DiFi human colon carcinoma cells. It is not yet known whether this also occurs in *in vivo* tumour models [171].

The quinazolines represent an important class of tyrosine kinase inhibitors, particularly with regard to the EGF receptor family. Because heterodimer formation between the EGF receptor and HER-2 leads to mitogenesis [129], inhibiting both tyrosine kinases may be more effective than blocking only the EGF receptor. PD 153035 inhibited both receptors [163], but HER-2 activity was not reported for ZD1839 or CP-358,774. At least three quinazolines are currently in clinical trials and results will provide important data on the therapeutic potential of this class of drugs. An open issue at this juncture is the pharmacokinetic profile in man; many of the quinazolines exhibit poor pharmacokinetics in animal models, although medicinal chemistry approaches have attempted to improve these properties. It remains to be determined in clinical trials whether the improvements are sufficient, and to gain an understanding of the pharmacokinetic/pharmacodynamic relationship.

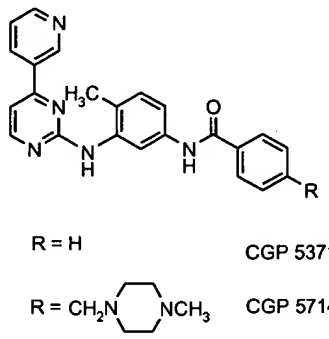
3.3 Substituted pyrimidines

Several substituted pyrimidines are in clinical development, or near clinical development. In addition to the quinazolines, they represent an important class of tyrosine kinase inhibitors. However, they may have broader application in their ability to inhibit, selectively or nonselectively, a greater number of targets. The substituted pyrimidines are comprised of pyrido-, pyrrolo-, pyrazolo-, pyrimido- and phenylaminopyrimidines. Novartis has a phenylaminopyrimidine in clinical development (CGP 57148) for the treatment of leukaemia and Parke-Davis has a number of pyridopyrimidines in late stage preclinical testing.

3.4 Phenylaminopyrimidines

Novartis synthesised a series of 2-phenylaminopyrimidines and pursued CGP 53716 as representative of the class [172]. This compound inhibited tyrosine phosphorylation of the PDGF receptor in *in vitro* and cellular assays with an IC_{50} of 0.1 μM in both types of assays. It was specific relative to the EGF receptor and a number of other tyrosine and serine/threonine kinases. The only other kinase it was found to potently inhibit was v-Abl. The cytoplasmic tyrosine kinase, v-Abl, was originally isolated as the product of the transforming gene of Abelson murine leukaemia virus [173]. Abl is also found as a fusion product with Bcr, resulting from a translocation between chromosomes 9 and 22 in human chronic myelogenous leukaemia [174]. CGP 53716 was orally efficacious at inhibiting the growth of v-*sis* and c-*sis* transformed BALB/c 3T3 cells in athymic mice, but did not inhibit

Figure 3: The structures of CGP 53716 and CGP 57148 (a phenylaminopyrimidine in Phase I clinical trials).



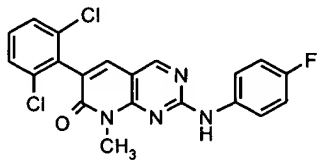
the growth of the EGF receptor-driven A431 cells as sc. tumours.

Novartis has pursued clinical studies with CGP 57148, a close analogue of CGP 53716 (**Figure 3**). This compound is also an inhibitor of the Abl and PDGF receptor kinases [175] with 30- to 100-fold selectivity when compared against the EGF, insulin growth factor-1 (IGF-1) and insulin receptor kinases. In intact cells, CGP 57148 inhibited v-Abl tyrosine kinase with an IC_{50} of 0.1 - 0.3 μM, with similar potency against the PDGF receptor. CGP 57148 exhibited antitumour activity *in vivo* in xenograft models. Doses of 3.13 - 50 mg/kg/day by ip. administration inhibited the growth of MuLV and v-*sis* transformed BALC/c 3T3 cells, with the highest dose almost achieving tumour stasis after 28 - 31 days of treatment.

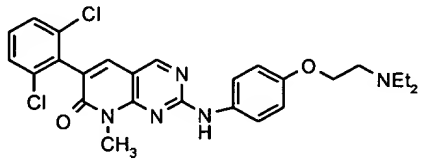
3.5 Pyridopyrimidines and pyrimidopyrimidines

Pyridopyrimidines, synthesised by Parke-Davis, are similar to quinazolines except that they have an additional nitrogen atom in the ring system. In the initial publication, a series of 7-aminopyrido[4,3-*d*]pyrimidines were compared for inhibitory activity on the EGF receptor [176]. As with the quinazolines, substitution in the meta position on the aniline ring increased potency compared to other positions, resulting in IC_{50} values of 0.01 - 1.5 μM. Methyl groups on the 7-amino group also resulted in nanomolar IC_{50} values [177]. Water solubility was increased while retaining inhibitory activity by the addition of a morpholine to the 7-amino group [178]. The position of the 'extra' nitrogen was also investigated [177]. Pyrido[3,4-*d*]pyrimidines were very similar to pyrido[4,3-*d*]pyrimidines at inhibiting the EGF receptor, but pyrido[2,3-*d*]- and pyrido[3,2-*d*]

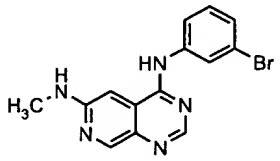
Figure 4: Representative pyridopyrimidines.



PD 173956



PD 166285



PD 158780

pyrimidines in this study were much less active, with the best IC_{50} being 3.1 μM. Subsequently, 6-phenyl substituted pyrido[2,3-*d*]pyrimidines were found to be potent inhibitors of the PDGF receptor, FGF receptor and Src [179]. Potency and specificity were altered by changing the substituents on the phenyl group.

Pyrimido[5,4-*d*]pyrimidines were synthesised to evaluate the effect of including an additional nitrogen atom in the ring system [180]. A series of analogues were prepared with electron-donating substituents in the 6-position and compared to quinazolines, pyrido[3,2-*d*]pyrimidines and pyrido[3,4-*d*]pyrimidines with the same substitutions. Although the pyrimido[5,4-*d*]pyrimidines were more potent in an *in vitro* EGF receptor kinase assay than pyrido[3,2-*d*]pyrimidines (1.5 *vs.* 7.6 nM for 7-amino-substituted), they followed the same pattern, i.e., there was no large increase in potency by adding methyl groups to the 7-amino, as was seen in the pyrido[3,4-*d*]pyrimidines. Crystal structure analysis indicated that the pyrido[3,4-*d*]pyrimidines had a conformational change to relieve the interactions between the hydrogen atoms on the carbon in the 5-position and the nitrogen in the 9-position. This

apparently allowed a 'supra-additive' effect with these compounds, such as was seen with quinazolines.

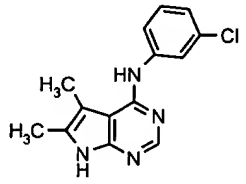
PD 158780 (**Figure 4**) is a pyridopyrimidine that inhibits purified EGF receptor tyrosine kinase with an IC_{50} = 8 pM and EGF receptor autophosphorylation in A431 cells at 13 nM [177,181]. Micromolar concentrations were required to inhibit PDGF- and bFGF-dependent processes in Swiss 3T3 cells, compared to low nanomolar for inhibition of EGF-dependent processes. PD 158780 was found to inhibit heregulin-stimulated phosphorylation in SKBR3 and MDA-MB-453 cells in the nanomolar range (IC_{50} = 49 and 52 nM). *In vivo* tumour growth was studied using A431 and MCF-7 xenografts, where 58% and 73% inhibition was achieved, respectively. A second pyridopyrimidine studied by Parke-Davis, PD173956, was found to inhibit c-Src activity with an IC_{50} of 26 nM, with selectivity compared to PDGF, FGF and EGF receptors (IC_{50} > 1 μM). *In vitro* antitumour cell activity was shown against colon cell lines with IC_{50} = 500 - 800 nM [181].

A third pyridopyrimidine, PD 166285, was shown to have impressive activity against PDGF, FGF and EGF receptors (IC_{50} = 39.3 - 98.3 nM) as well as Src family members, for which it was most potent. An IC_{50} = 8.4 nM on c-Src and IC_{50} < 1 nM on Fyn, Lyn and Lck was observed [183]. Broad *in vivo* activity was observed with PD 166285 against NIH 3T3/PDGF transfectants, C6 (rat glioma), HT29 (human colon) and SKOV-3 (human ovarian) cells. Recently, Hamby *et al.* [184] conducted SAR studies around the C-2 amino and C-6 aryl positions of the pyridopyrimidines and found compounds with improved potency, solubility and bioavailability, important considerations for clinical development candidates. 3',5'-Disubstituted phenyl compounds were selective inhibitors of FGF receptor tyrosine kinase with an IC_{50} of 0.77 μM compared to > 50 μM for PDGF and EGF receptors and c-Src.

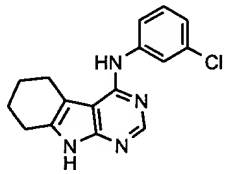
3.6 Pyrrolopyrimidines and pyrazolopyrimidines

Pyrrolo- and pyrazolopyrimidines have a five-membered ring fused to a six membered ring, instead of two six-membered rings. Novartis designed pyrrolopyrimidines to fit its pharmacophore model for the binding of dianilinophthalimides [185] into the ATP binding site of the EGF receptor [186]. In *in vitro* assays, CGP 59326, CGP 60261 and CGP 62706 (**Figure 5**) were potent for inhibition of the EGF receptor with IC_{50} values less than 30 nM and were specific relative to c-Src, v-Abl and protein kinase C.

Figure 5: Representative pyrrolopyrimidines.



CGP 59326



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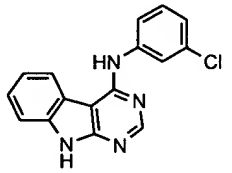
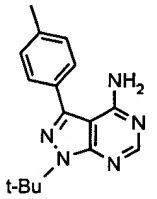


CGP 62706

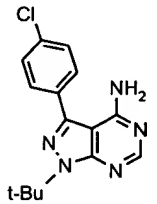
They also inhibited EGF receptor phosphorylation in EGF-stimulated BALB/c 3T3 cells, but not PDGF receptor phosphorylation with PDGF treatment. CGP 59326 was efficacious as a single agent and in combination with cytotoxic agents in several EGF receptor expressing-human xenograft models. This compound is close to entering clinical trials [5].

Pyrazolopyrimidines have one more nitrogen atom in the five-membered ring than pyrrolopyrimidines. Pfizer has used two such compounds to study the role of Src family members, Lck and Fyn, in T-cell activation [187]. PP1 and PP2 (**Figure 6**) inhibited Lck and Fyn in *in vitro* assays with IC_{50} values around 5 nM. They did not inhibit ZAP-70 or JAK2, and potency on the EGF receptor was about 120- to 200-fold less than on the Src family members. PP1 also inhibited antiCD3-stimulated tyrosine phosphorylation and proliferation of human T-cells. Novartis is optimising pyrazalopyrimidines as EGF receptor tyrosine kinase inhibitors [186].

Figure 6: Structures of pyrazolopyrimidines.



PP1



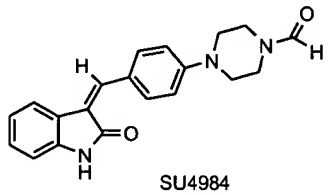
PP2

The substituted pyrimidines are beginning to enter clinical testing (e.g., CGP 57148) and it is likely that several additional molecules will enter clinical development soon. Unlike the quinazolines, which target only the EGF receptor family, substituted pyrimidines may have broader application. Activity has been observed against PDGF receptor and Src family members. In addition, they have the potential to hit a number of targets, as exemplified by the activity of PD 166285 against PDGF, FGF and EGF receptors and c-Src. If such a compound exhibited a good toxicological profile, it may have potential for broad applicability in treating cancer (see section 4). They may also be useful therapeutics for restenosis, where the PDGF and FGF receptors both play a role.

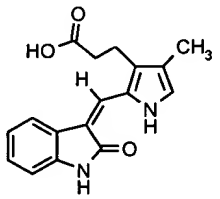
3.7 Indolin-2-ones

In addition to the substituted pyrimidines, the 3-substituted indolin-2-ones represent a second class of tyrosine kinase inhibitors that have the potential for broader target activity. Three 3-substituted indolin-2-ones have been described (**Figure 7**) by SUGEN, one of which is in clinical development. SU4984 and SU5402 were co-crystallised with the FGF receptor type 1 kinase domain [188]. Analysis of the structures showed that these compounds bind to the ATP binding site with the indolin-2-one ring system in the site for the ATP adenine. The side-chains on the indolin-2-one core conferred specificity to the FGF receptor tyrosine kinase. SU4984 inhibited the PDGF, EGF and insulin receptors, as well as the FGF receptor. In the crystal structure, hydrogen bonding

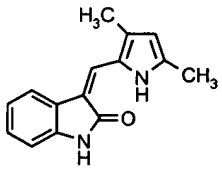
Figure 7: Structures of 3-substituted indolin-2-ones.



SU4984



SU5402



SU5416

between the indolin-2-one core and the enzyme was found only to peptide backbone atoms that are conserved in tyrosine kinases. SU5402, which specifically inhibits the FGF receptor, formed additional hydrogen bonds from the carboxylic acid group to Asp 568, which is specific for the FGF receptor.

SU5416 is currently in clinical development by SUGEN as an anti-angiogenic agent. It is a potent Flk-1 tyrosine kinase inhibitor [189; Fong *et al.*, manuscript in preparation] with an IC_{50} of approximately 1 μM in a cell-based assay. It has 20-fold selectivity against the PDGF receptor and 100-fold selectivity against the EGF receptor, HER-2, and IGF-1 receptor. Suprisingly, it does not inhibit FGF receptor kinase activity despite its similarity to SU5402. Apparently, substitutions on the pyrrole ring are important for conferring specificity. SU5416 inhibited the proliferation of endothelial cells stimulated with VEGF, with an IC_{50} of 40 nM. The compound has also shown broad-range activity in inhibiting xenografts in athymic mice at doses of ≤ 25 mg/kg/day. Additional indolin-2-one compounds with different target profiles are currently in preclinical development at SUGEN.

4. Future directions

With the discovery of oncogenes, particularly of the tyrosine kinase family, as components of signal transduction pathways came the speculation that inhibitors may provide therapeutic benefit to cancer patients. Since that time, it has become clear that tyrosine kinases play a role in diseases outside of cancer, which might benefit from drugs that target these enzymes. For example, it is clear that anti-angiogenic tyrosine kinase inhibitors will have broad applicability in diseases such as rheumatoid arthritis, ocular diseases of neovascularisation (macular degeneration, diabetic retinopathy), psoriasis and restenosis [190].

Over the past fifteen years, several issues have been raised that posed potential roadblocks to the development of such agents. Although most questions are being addressed in the context of treating cancer patients, the answers will certainly have implications for the other diseases where kinases play important roles. The first question that was raised was one of expression, comparing normal cells or tissue to their cancerous counterparts. It was reasoned that if expression could be found to be limited to the cancerous cells, the likelihood of success in developing a tyrosine kinase inhibitor might be greater. Many studies have examined the expression of tyrosine kinases in normal *versus* cancerous tissue. Indeed, some were found to be overexpressed compared to normal cells, but few have been found to be solely expressed in cancer cells. Therefore, there has been scepticism concerning the ability of tyrosine kinase inhibitors to selectively act on tumour cells, and discussion about safety and side-effects arising from the potential to also act on normal cells. This has been less of an issue in the last few years, as it has been learned that normal cells have multiple, redundant, signalling pathways, but that diseased cells usually have one primary pathway causing the altered signalling.

A second issue that has been widely debated is the potential for general toxicity of tyrosine kinase inhibitors that were designed to be competitive with ATP. Two arguments have fuelled this debate. First, kinases are required for normal cellular functions, such as proliferation, migration, metabolism and survival. The catalytic domains of protein kinases are highly conserved and it was reasoned that compounds must bind similarly into the binding pocket of all ATP-utilising enzymes and, thus, could not be expected to be specific. Therefore, ATP-competitive inhibitors that work on kinases in diseased cells would interfere with the activity of other crucial enzymes in non-cancerous cells and tissues. Secondly, the concentration of ATP inside the cell is sufficiently high and the binding affinity (Km) for ATP is sufficiently low that it would be impossible to identify inhibitors that could overcome both of these properties. Indeed, the design of early kinase inhibitors was based on the structure of phosphotyrosines (tyrphostins). These were designed to be competitive for substrate rather than ATP.

The above has led to the general belief that it would be very difficult, if not impossible, to develop potent, ATP-competitive, selective tyrosine kinase inhibitors. Several examples now exist of tyrosine kinase inhibitors that are very selective, as discussed earlier. Quinazolines have been shown to be competitive for ATP binding to the EGF receptor [165]. It is likely that quinolines and quinoxalines also compete with ATP. It is unclear how such similar compounds can be specific for different receptors. As new kinases and compounds are co-crystallised, the fine differences in receptor specificity may be better understood and allow for the development of new compounds through computer modelling and drug design. For example, crystallisation studies confirmed the binding of SU5402 in the ATP-site, yet it is an example of a relatively selective tyrosine kinase inhibitor with activity on the FGF receptor and the VEGF receptor, Flk-1 [188].

Most small molecule inhibitors currently in clinical development were purposely designed to be very specific. In comparison to conventional chemotherapeutic agents, they had better toxicological profiles in animal models and their safety is now being studied in man. If their safety is confirmed, it is likely that less selective tyrosine kinase inhibitors that have activity on more than one kinase, such as the substituted pyrimidines or indolinones, will be tested as well.

It is important to point out that the long-term consequences of inhibiting tyrosine kinases have not been studied at this time. Given their role in development, it is likely, at the very least, that tyrosine kinase inhibitors would have effects on developing embryos and thus might be contraindicated during pregnancy. This is supported by studies in mice where tyrosine kinases have been genetically altered *via* targeted mutagenesis, resulting in embryonic death.

The future of tyrosine kinase inhibitors as therapeutic agents will be clearer in a few years, when results of the current clinical trials become known. Based on preclinical results, the new generation of tyrosine kinases inhibitors look very promising for the treatment of cancer and other proliferative diseases.

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